

Title: ANTI-PARATOPIC ANTIBODY AS AN IMMUNOGEN

Field of the Invention

This invention relates to immunology and more particularly to a method of manufacture of immunogenic, compositions, to immunogens manufactured by the method, and to antibodies manufactured therefrom.

Background Art

An immunogen is a molecule capable of eliciting an immune response in a vertebrate. The response elicited is believed to be determined by topographical shape characteristics of the immunogen. Immunogens are also called antigens i.e. ANTIBody GENERators because one aspect of the induced response involves the production of antibody molecules whose function is to lock onto the immunogen. Those areas of the immunogen to which the antibody molecule binds are variously referred to as the antigenic determinants, epitopes or haptens. The last

term, namely hapten, is generally associated with the term carrier and this term refers to that part of the immunogen/antigen which interacts with cellular components of the vertebrate immune system.

5 These regions on the immunogen and the names used to define them should not be regarded as absolute. Thus the genus of vertebrates has immune systems which will recognize immunogens; but not all species necessarily recognize the same molecular areas as being haptenic areas or carrier areas. Within a species, the
10 recognition of haptenic molecular areas can only be determined experimentally. Thus, mice will not necessarily process immunogens in the same way as would, for example, the immune system of Man. Furthermore,
15 within a species, individual specimens will not respond to the same degree. This is because the immune response to an immunogen has a genetic (hereditary) component. Thus, some individuals will respond better to an immunogen while others may not respond at all.

20 The immune response to an immunogen is an integrated phenomenon in that a class of white blood cells called T lymphocytes, for example, reacts with the carrier determinants which in turn allows a class of white blood cells called B lymphocytes to transform and
25 start producing antibodies to the antigenic determinants.

Each cell recognizes only one determinant and each antibody producing B cell (plasma cell) generates only

antibody molecules of one given specificity. Hence, the immune system is said to be highly specific. Upon stimulation, these plasma cells multiply and thereby give rise to a clone of identical antibody secreting cells. If it were possible to isolate these identical antibody secreting cells, they would be referred to as monoclonal and the antibodies referred to as monoclonal antibodies.

Figure 1 is a diagrammatic illustration of the response of a mouse to an immunogen/antigen. Under normal conditions each monoclonal antibody generated by the mouse in vivo mixes with other monoclonal antibodies so that a polyclonal antibody response eventuates.

Each antibody comprises a glycoprotein molecule. The portion of an antibody molecule embodying the characteristic of shape or molecular topography, or code sequence which enables it to bind and so for example neutralise the antigenic determinant or epitope of an antigen is known as a "paratope". The paratope is conceptually a molecular region of a shape complimentary to the epitope or to a part of the epitope of the antigen and is thought to reside in the so called hypervariable region of the antibody glycoprotein molecule.

Antibody producing lymphocytes are present in high concentration in the spleen but antigen reactive spleen lymphocytes cannot readily be cultured in isolation.

However, monoclonal antibodies may be manufactured and isolated therefrom by use, for example, of techniques of hybridoma technology. In one such technique mice are first exposed to an antigen whereby the mouse develops antibodies. With reference to Fig. 2, spleen cells of the immunised mouse are fused with mouse myeloma cells. The growth of hybrid cells is promoted and the hybrids are screened for specific antibody secretion. Those useful are cultured or undergo further genetic stabilisation procedures. By this means specific monoclonal antibodies may be produced and isolated.

Selected antibodies, or mixtures thereof such as are produced in the method of Fig. 2, may be used to neutralise an antigen in an organism, a paratope of each antibody in effect forming a complex with an epitope of the antigen.

In anti-idiotypic immunology a second stage process shown in Fig. 3 is involved. Mouse 1 is first immunised with an antigen. Thereby giving rise to several clones of antibody producing cells. One cell line is chosen on the basis of the characteristics of the generated antibody and the antibody is referred to as Abl. Abl is then used to immunise a second mouse - mouse 2. The latter must have a genetic constitution very similar to, or identical with that of mouse 1. Mouse 2 generates monoclonal antibodies to Abl, a subset of which may be directed against the paratope of Abl. All the antibody

subsets generated by mouse 2 against Abl may be referred to as Ab2 though the Ab2 subset specific for the paratope of subset 1 is sometimes referred to as Ab2 beta. The second mouse monoclonal antibody, Ab2, has an anti-paratope, that is to say having a molecular portion with a shape characteristic complementary to the paratope of the first antibody. If the epitope of the original antigen is considered to be "mould positive", the paratope of the monoclonal antibody Abl can be considered to be a counterpart or "mould negative" and the paratope of the anti-Abl antibody that is the paratope of the Ab2 monoclonal antibody can be considered to replicate the "mould positive". It will be understood however that in each case the replication is not exact. When used in a vaccine, the second monoclonal antibody, Ab2, functions as a harmless immunogen which stimulates production of AB3 antibodies in the vaccinated animal effectively producing immunity to the first antigen.

Anti-idiotypic vaccines are designed to be an interspecies approach so as to identify epitopes which induce neutralising antibodies in genetically diverse population. The approach requires that the anti-idiotypic vaccine candidates (Ab2) be inoculated into populations as diverse as sheep, chimpanzees and rabbits followed by antigen challenge to determine if the Ab3 carries the neutralising characteristics of the

Abl. If the challenge is successful Abl is further studied as a possible vaccine. If challenge is unsuccessful, the antibody is disregarded for its role as a vaccine. In this approach, the animal studies are meant to simulate the genetic diversity found in Man. The epitopes are therefore exclusively public in that they are capable of generating neutralizing responses in a number of different species.

Disclosure of the Invention

10 According to one aspect of the present invention consists of a method of treating an animal comprising the steps of:

(1) selecting from a pool of antibodies occurring in a first species of vertebrate a prototypic set the members of which are antibodies with specificity for a specific antigen or antigen epitope expressed in one or more members of the first species, and not expressed in members of a second species;

(2) utilizing one or more members of said prototypic set, or paratopic fragments thereof, as an immunogen in a host of the second species, wherein said second species is selected such that the immune system of said second species does not recognise the specific antigen or antigen epitope uniquely expressed in one or more members of said first species, or said paratopic set or paratopic fragments thereof are utilized as an

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first species, or said paratopic set or paratopic fragments thereof are utilized as an immunogen in an in vitro incubation system comprising cells derived from the same or a different species to produce one or more antibodies having a characteristic which is anti-paratopic with respect to the antigen or antigen epitope expressed in one or more members of said first species but not expressed in members of the second species; and

(3) selecting and purifying said anti-paratopic antibodies produced according to step 2.

In a preferred embodiment of the invention the anti-paratopic monoclonal antibodies are used to immunize a different member of the first species as that from which the prototypic set was selected.

For preference the pool of antibodies consists of naturally occurring human antibodies.

The prototypic set is a set of antibodies selected on the basis of effectiveness against a particular antigen, or epitope thereof, for example is a set of human antibodies selected from humans carrying antibodies resulting from exposure to HIV.

The antibodies, or paratypic paratope bearing segments of them, are utilized as a immunogen in a mouse host to produce mouse antibodies having anti-paratope characteristics.

The mouse antibodies are then screened first for

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their ability to specifically bind antigens or antigen epitopes in relation to the first species and then in respect of their effectiveness for inducing antibodies which bind the HIV in other members of the first species.

5 It will be understood that the response generated against the antigen or antigen epitope under consideration would be uncommon in nearly all species except the first species from which the prototypic set of antibodies was selected.

10 Brief Description of the Drawings

Figure 1 is a diagrammatic illustration of the response of a mouse to an immunogen/antigen.

Figure 2 is a diagrammatic representation of monoclonal antibody production.

15 Figure 3 illustrates anti-idiotypic antibody Ab2 production.

Figure 4 is a schematic representation of the method of manufacture of anti-paratopic antibodies according to the invention.

20 Figure 5 (I) illustrated a general procedure for the purification of HIV positive human antibodies.

Figure 5 (II) illustrates a general procedure for the purification of HIV antigen specific human antibodies.

25 Figure 6 (a) illustrates purification of human IgG prior to delineation into HIV/HIV antigen specific antibodies.

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Figure 6 (b) illustrates purification of human IgA prior to delineation into HIV/HIV antigen specific antibodies.

5 Figure 6 (c) illustrates purification of human IgM prior to delineation into HIV/HIV antigen specific antigens.

Preferred embodiments of the invention have a number of advantages over the prior art.

10 Firstly, the invention produces a mouse anti-paratope which is a counterpart of a naturally occurring human antibody paratope for a specified antigen. Upon inoculation the mouse anti-paratope monoclonal antibody produces in a human an antibody bearing a replica of the naturally occurring human
15 paratope.

20 In the prior art there was produced a mouse anti-paratope which was a counterpart of an artificially generated mouse antibody. Such a mouse anti-paratope monoclonal antibody would produce in a human an antibody bearing a replica of an artificially created mouse paratope (in contrast to a human paratope) and which may not be as effective in binding the specific antigen in a human. In relation to the prior art the invention does
25 not rely upon the assumption inherent within the prior art that the mouse processes antigen in exactly the same way as humans.

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Secondly, the present invention has as its basic

premise the observation that epitope presentation within
a particular species is unique to that species. The
prior art technology looks to immuno-dominant epitopes
common to all species and aims to produce mirror images
to them. Epitopes selected in the prior art technology
are identified using empirical approaches and numerous
algorithms have been used to predict antigenic
sequences. In the present invention, antigenic
sequences necessary to produce neutralizing epitopes are
believed to be both linear and assembled. Antigen
presentation is a multifactorial operation involving
several host immune components. Hence, the basic
premise of the present invention is that epitope mapping
algorithms while applicable do not identify all epitopes
of immunological significance. It is in this area which
the present invention is focused. The host immune
system has a role in the amplification and display of
the total repertoire of epitopes of the invading
immunogen. The present invention capitalises on this
factor whilst prior art technologies have tended to
approach the problem from a more conventional
anti-idiotypic approach.

Thirdly, in comparison with the prior art scheme
illustrated in Fig. 3, the invention provides a more
direct general route shown schematically in Fig. 4 to
the production of an anti-idiotypic antibody.

Fourthly, since preferred embodiments of the

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invention use widely available and naturally occurring i.e. endogenous, antibodies as the starting material rather than antigens, the process is expected to be less costly to conduct.

5 Fifthly, the process is safer to conduct than processes requiring handling for example of potentially harmful virus antigens.

10 Finally, both the present invention and anti-idiotypic technology use hybridoma technology, protein chemistry and immunology. When testing the putative vaccine, however, anti-idiotypic vaccines have to be tested for complimentary and efficacy in several animal species e.g. rabbits, sheep, baboons or chimpanzees etc. This is necessary to compensate for
15 the interspecies approach used to generate and test the vaccine candidate. This is a relatively long and time consuming step. The vaccine candidate produced in the present invention, however, has to be tested primarily for complementary within the species to be immunized.
20 It is designed to be primarily an intra-species approach. Accordingly, from a small pool of infected individuals either immune to a particular infectious agent or carrying neutralizing antibodies to it and using standard techniques (or minor technological
25 variants) to produce anti-paratopic antibodies, it is possible using the present invention to generate specific vaccines against said infectious agent. These

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vaccines can then be used to treat a small number of infected individuals or to immunize an entire population of individuals prone to infection by said infectious agent.

5 The particular advantage of the present invention is that in the case of some viruses, for example AIDS, there are so many epitopes some of which are protective, some of which are suppressive, some of which are dominant and some which have no affect on the immune
10 system. In the present invention, selection of the antibody is not dependent on the epitope. Instead, selection is based on whether the antibody produced is neutralizing or not. In contrast, in prior art
15 approaches, antibody selection depends on an epitope being common to a variety of different species. For example, if an antibody works in rabbits but not in guinea pigs, it is discarded. The result being to reject it but in doing so, the very epitopes which could protect human populations may be lost. This is overcome
20 by the present invention because reliance is not placed on epitope recognition between species.

Best Modes of Performing the Invention

25 An embodiment of the invention will now be described by way of example only. The embodiment concerns the manufacture of a vaccine to confer immunity against Acquired Immune Deficiency Syndrome (AIDS). The invention is not, however, limited to use for production

of any particular vaccine, and has uses other than for the production of vaccines.

The manufacture may be considered as involving the steps of:

- 5 (1) selecting a prototypic set of antibodies;
 (2) preparing one or more immunogens therefrom;
 (3) inoculating hosts with one or more immunogens;
 (4) generating a monoclonal antibody pool from each host;
10 (5) screening the monoclonal antibody pools;
 (6) testing the screened antibodies for effectiveness as a vaccine.

15 In the example under consideration the first stage is to select from the pool of human antibodies a prototypic set, in this case a set of immunoglobulins which effectively bind the aetiologic agent for Acquired Immune Defficiency Syndrome (AIDS). The generally accepted aetiological agent for AIDS is currently known as Human Immunodeficiency Virus hereinafter referred to
20 as HIV.

That is accomplished by obtaining human immunoglobulins from individuals exposed to HIV. About 75% of such individuals have antibodies to HIV.

25 The antibodies from these individuals are screened for effectiveness in binding HIV antigens and/or antigenic fragments. Those antibodies effective at this function are retained as members of the prototypic set

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i.e. they are a subset of the pool of human immunoglobulins.

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~~If~~ desired the retained immunoglobulin members so selected may be purified and used directly in step (3).

- 5 Preferably, however, in a second step the human immunoglobulins ("Ig") are subdivided into classes G, A, M, D, E (to use the WHO designation) and more particularly identified in Table I.

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Table 1

PHYSICAL PROPERTIES OF MAJOR HUMAN IMMUNOGLOBULIN

CLASS IN SERUM

WHO Designation	IgG	IgA	IgM	IgD	IgE
Sedimentation Coefficient	7S	7S, 9S, 11S*	19S#	7S	8S
Molecular Weight	150,000	160,000+ dimer	900,000	185,000	200,000
Number of Ig Units	1	1-2	5	1	1
Number of Antigen Binding Sites	2	2-4	10	2	2
Identity of Heavy Chain	(gamma)	(alpha)	(mu)	(delta)	(epsilon)
Carbohydrates Content	3	8	12	13	12
% Total Immunoglobulin in normal human serum	80	13	6	0-1	.002
Concentration range in normal human serum	8-16 mg/ml	1.4-4 mg/ml	0.5-2 mg/ml	0-0.4 mg/ml	17-450 mg/ml

* IgA dimer found in mucosal (secretory) immune system. It is complexed with a secretory component (MW=60,000) and J chain (MW=15,000).

IgM contains J chain.

Source: I.M. Roitt, Essential Immunology, 4th Ed. Blackwell 1980

More desirably still the immunoglobulins are further divided into sub-classes, for example, IgG being divided into four sub-classes, IgA being divided into two sub-classes and IgM into two sub-classes. In the preferred embodiment each of sub-classes IgG 1-4, IgA 1-2 and IgM 1-2 are purified and isolated from each other. IgD and IgE sub-classes are present in immunoglobulin in small concentration and their inclusion is optional.

The human IgG/A/M is drawn from three main groups affected by AIDS viral infection, viz

- male homosexuals
- bisexual/female/heterosexual AIDS carriers
- haemophilics

Blood plasma is heated to 56°C to kill the virus. Cellular components and serum debris are removed either by aspiration of the serum component or by centrifugation (in the case of plasma).

Human IgG can be purified free of all non-IgG contaminants by affinity chromatography. Other procedures such as ion-exchange chromatography may be used but affinity chromatography is preferred for speed and selectivity. More particularly, purification is generally effected by means of chromatography using PROTEIN-A SEPHAROSE beads (obtainable from e.g. Pharmacia Biotechnology Pty Ltd.).

Sub-classes of IgG may also be isolated by chromatography.

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In a similar manner human IgA purification may be carried out by anti-IgA affinity chromatography.

Human IgM may be purified by a combination of

- (a) Protamine sulphate chromatography, and
- 5 (b) Column chromatography, or
- (c) IgM affinity chromatography.

The purified prototypic immunoglobulins set may be used directly as an immunogen for inoculation of mice in stage 3.

- 10 ~~Alternatively, the Ig sub-classes may be screened to select antigen specific antibodies for use as the immunogen. In this case, the Ig sub-classes are next screened for effectiveness against HIV antigen to select the most effective sub-classes in binding the antigen.~~
- 15 More preferably, the antigen is first divided into sub-classes known as p18, p24, gp41, p55, gp120 and gp160. These antigen sub-classes differ from each other in molecular structure and can be separated by
- 20 SDS-polyacrylamide gel electrophoresis. Each Ig sub-class is then screened against each antigen sub-class to select the most effective Ig's.

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HTLV III - HUMAN SERUM Ig PARATOPE GRID

Human Serum Ig's*	No. of Epitopes	IgG (80%)				IgA (13%)		IgM (6%)		Antigen Specific Human Paratope
		1 (65%)	2 (23%)	3 (8%)	4 (4%)	1 (80%)	2 (20%)	1	2	
HTLV III Antigens										
p24	2									16
gp41	4									32
p53	5									40
gp120	12									96
gp160	16									128
Carrier Specific Idiotypes		39	39	39	39	39	39	39	39	
Total Number per Ig Class		166				78		78		
% Spread of Idiotype		50%				25%		25%		

* IgD and IgE not Included

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With reference to Table 2, there is shown a "paratope grid". If it is assumed that there is one antigenic group anchored to a ten thousand dalton carrier group then the total number of antigenic groups (epitopes) available among the five antigenic sub-classes would be thirty nine. With eight potential antibody classes in the grid that can respond to the thirty nine antigens the total number of possible antibodies carrying paratopes specific for HIV is 312. Put differently, there are on average thirty nine HIV paratopic bearing human immunoglobulins per immunoglobulin sub-class. Thus, for example, it might eventuate that human IgG1 has specificities for all thirty nine epitopes ("haptens"), i.e., there would be thirty nine IgG1 molecules all absolutely identical except for one feature namely their Fab paratope would be different.

In the third step of the embodiment, one or more members of the prototypic set are used as an immunogen in a non-human host for example by being injected into a mouse. The one or more members are preferably the most effective of the immunoglobulin sub-classes. The criteria of effectiveness may be effectiveness against a specific antigen or effectiveness against a spectrum of antigen sub-classes or other criteria.

Human antibodies are excellent immunogens when injected into mice. The antigenic sites on the human antibody molecules are spread right across the length of

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the molecule from the NH_2 terminii--ie the Fab end to the
carboxylic acid terminus --ie the Fc end. The Fab NH_2
end carries the paratope. Other antigenic components of
the Fab are present for structural or "carrier" purposes.

- 5 For the purposes of the vaccine the Fc exclusively
exhibits "carrier" as opposed to paratope antigens.

Immunization studies have demonstrated that not all
the antigenic sites on the intact human immunoglobulin
molecule are of equal value in that a greater proportion
10 of induced antibodies tend to be directed against the Fc
region. This phenomena is described as antigenic
competition or more accurately as intramolecular antigenic
competition. When developing an anti-paratopic antibody,
however, the part of the molecule of most interest is the
15 Fab area that is to say the paratope bearing region. A
simple way to overcome the problem of Fc dominance is to
enzymatically cleave the immunoglobulin molecule and
isolate the Fab fragment. When used to immunize a mouse
this will cause all the induced immunoglobulins to be
20 directed against the Fab fragment. A subset of the anti-
Fab antibodies generated by the mouse, irrespective of
whether an intact immunoglobulin molecule or a Fab/F(ab)'₂
fragment has been used, will be directed against the
internal idiotope i.e. paratopic image of the human
25 immunogen. Thus, the member of the anti-HIV prototypic
set used as an immunogen in the mouse may be either (a)
the mixed intact human immunoglobulin specific for the

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AIDS virus, (b) selected classes or sub-classes of the intact immunoglobulin, (c) a Fab/F(ab)'2 fragment of one or a combination of the AIDS specific immunoglobulins or (d) a Fab/F(ab)'2 fragment of one or a combination of the AIDS specific immunoglobulins complexed to carries eg. Keyhole Limpet Haemocyanin or human albumin.

The stage of preparation of immunogen may thus include enzymatic digestion or chemical cleavage of the human anti-HIV immunoglobulin and conjugation of the Fab/F(ab)'2 to microspheres or the like.

As will be apparent from the foregoing, it is conceivable that when injected into the mouse, the HIV IgG1 subgroup could provide all the relevant paratopes on one type of carrier. This regime would favour the generation of anti-idiotypes in the mouse (as opposed to the generation of anti-"carrier" molecules).

There is a possibility though of inter-molecular antigenic competition so that only a small variety of the human paratopes directed against HIV will end up being antigenic in the mouse. If this occurs then there are various ways of proceeding:

- (i) after the screening step those paratopes that are dominant could be isolated from the immunogen population and a second immunization carried out to develop mouse anti-paratope antibodies to the remaining paratopes.
- (ii) the anti-idiotypes/idiotypic reagents arising from the first immunization could be screened and tested to see

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TABLE 3

STANDARD METHOD		"LOTTO"	
one-hit		(multi-chance)	
(i) immunize			
(ii)	4 wks later boost	(ii)	2-3 days later boost
(iii)	4 days later	(ii)	2-3 days later
spleen cell prepn		cell prepn	
(iv)	Hybridoma productn	(iii)	Hybridize
(v)	Preliminary screen	(iv)	Preliminary screen
(vi)	CLONE	(v)	Clone

The Fab pool may be screened by conventional means as shown in Table 4:

TABLE 4

SCREENING FOR FAB POOL

Antigen	Mc	Ab	Configuration		(+Ve/-Ve)	
	1	2	3	4	5	6
Bence-Jones	+	-	+	-	+	-
Human Ig	+	+	-	+	-	-
Immunogen	-	-	+	+	-	+
ACTION						
Discard/Retain	D	D	D	D	D	R

The anti-idiotypic pool may be screened by conventional means. For example, HIV on a tray is mixed with human anti-HIV antibodies before and after incubation with mouse HIV idiotypic complexed to microsphere/eupergit spheres, then chased with anti-mouse Ig-PO, +Ve is discarded, and -Ve is retained.

Alternatively, HIV on beads is mixed with human anti-HIV PO-enzyme + mouse HIV idiotypic +Ve response is discarded.

As will be appreciated by those skilled in the art the antibodies may be selected from a pool occurring in a different species of vertebrate and the prototypic set may be selected from effectiveness against a different antigen. The antibodies may not be free in plasma but may be bound to cells (e.g. B cells) or may exist as immune complex. The prototypic set may be divided into members using different criteria from that exemplified.

Other methods may be used for separation such as use of dyes bound to inert supports, or the use of monoclonal antibodies, etc. and purification of the immunogen without departure herefrom.

The immunogens, or fragments thereof may be utilized in host species other than mice.

The antibodies so obtained may be used in various ways for example for immunization of the vertebrate from which the antibodies were obtained, in test methods and for other purposes.

The invention will now be described more specifically by way of the following Examples.

PREPARATION OF HUMAN IMMUNODEFICIENCY VIRUS SPECIFIC HUMAN ANTIBODIES

5 The desired antibody is designated as mouse Ab2. However, the production of mouse Ab2 is dependent upon the prior availability of a first antibody (Abl) of interest. Presently, there are two sources of Abl. The first source is the mouse Abl produced in vitro, and the second is the
10 human Abl to HIV.

 There are several good reasons to choose the human Abl as the immunogen. Little is known about the epitopes that are relevant in this disease (HIV), other than the identification of the CD4 antigen on the T cell, and those
15 epitopes on the envelope. It has also been postulated that transmembrane proteins may be of importance based on hydrophilicity indices.

 The issue about epitopes is that they are primarily linear sequences. Little is currently known about
20 conformational epitopes, neotypes or cryptotopes. A prior knowledge of epitopes of interest is unimportant when human antibodies are being used.

 Secondly, the virus is known to be specific for man which is a good reason to use antibodies induced in situ
25 as the starting material to induce the production of the Ab2. It is possible that there are specific epitopes which are of some importance to the human situation which

will not be seen in other species.

A. PREPARATION AND PURIFICATION OF HIV ANTIGENS.

Native and recombinant antigens can be purified by affinity chromatography using human antibodies or
5 antibodies from another species such as mouse monoclonal antibodies specific for the HIV antigens. By way of illustration the procedure described will be that using human antibodies. There is very little difference between the two approaches though the benefit is that with the
10 appropriate mouse monoclonal antibodies specific antigens can be purified only if the antigen source is the native one. However, if the antigen source is a recombinant one then human antibodies will allow for the specific purification of the recombinant antigen. When human
15 antibodies are used the steps involved are
(1) the preparation of human IgG from HIV infected individuals.
(2) the preparation of the human antibody (IgG) column and
20 (3) the purification of the viral antigens using the aforementioned column.

1. Preparation of human antibodies.

According to this procedure human antibodies were first purified by either hydroxyapatite chromatography,
25 ion-exchange chromatography (DEAE-cellulose) or protein-A affinity chromatography. By way of example the method for the purification described is that of protein-A agarose

column chromatography.

Pooled human sera was obtained from patients positive for the AIDS virus as determined by both an AIDS antibody ELISA assay and subsequently confirmed by the Western Blot assay. Prior to use the serum had been heat treated (56°C for 30 mins). A 2.0ml protein-A agarose column was washed with 20 ml of the Monopure binding buffer (Pierce). 4mls of the pooled serum was diluted with 8mls binding buffer and centrifuged (200xg:10 min:RT). The supernatant was applied to the column, allowed to percolate through and exhaustively washed in the binding buffer. The human IgG was specifically eluted using the commercially obtained elution buffer (Pierce). Following dialysis and concentration, the A_{280} data was used to determine the concentration of protein which was calculated to be 30 milligrams as determined by the $E^{1\%}_{1\text{cm}}=1.43(280\text{nm})$. Western Blot and ELISA data confirmed the presence of HIV specific antibodies in the IgG fraction purified in this manner.

2. The preparation of the IgG affinity column.

30 mgs of the human IgG was equilibrated in the coupling buffer (0.1M NaHCO_3 pH8.3 + 0.5M NaCl) and mixed with 4 gms CnBr-Sepharose 4B (Pharmacia) which had been pre-washed in 1mM HCl, swollen and equilibrated in the coupling buffer. The mixture was mixed end-over-end in a sealed coupling vessel (2hrs, RT). Unreactive groups on the matrix were blocked using 0.2M glycine in the

coupling buffer (16 hrs, 4°C) and the ensuing IgG-Sepharose matrix exhaustively washed in high salt and variable pH buffers prior to the purification of the HIV antigens.

- 5 3. The purification of the native/recombinant antigens. By way of illustration, the method described is that for the recombinant HIV antigens in particular recombinant 'gp120'.

Sub genomic clones of HIV cDNA encoding gp120, gp41, p24 and p18 were cloned and amplified in E. coli using λ gt11. The E. coli lysates were screened with in-house and by commercial HIV antigen ELISA's.

Radioimmunoprecipitation studies confirmed the presence of recombinant HIV antigens and the molecular weights of the recombinant antigens were as predicted e.g. 60kD for the recombinant 'gp120'.

Following precipitation of E. coli antigens with $(\text{NH}_4)_2\text{SO}_4$ the supernate was concentrated (Amicon) dialysed against distilled water and then against 0.05M Phosphate buffer pH7.2 (16hrs, 4°C). 40 mls of the dialysed concentrate was combined with approximately 2 ml of the IgG-Sepharose and the mixture incubated end-over-end for 2 hrs (RT). The matrix was exhaustively washed and the recombinant protein eluted using 4M MgCl_2 , pH 8.3. The presence of recombinant antigen was confirmed as outlined above.

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B. THE PURIFICATION OF THE HUMAN HIV SPECIFIC ANTIBODIES.

The purification of HIV specific human antibodies involved two steps. These are outlined below.

- i The preparation of the HIV antigen column.
- 5 ii The preparation of the HIV specific human antibodies.
1. Preparation of the HIV antigen-Sepharose column.

7.5 mls of the eluted protein was mixed with 2 gms swollen, pre-washed and appropriately equilibrated CnBr-Sepharose (pH8.3). The mixture was mixed
10 end-over-end (2hrs, RT). Unreactive sites were blocked using 0.2M glycine (16hrs, 4°C) and the matrix exhaustively washed as outlined for the IgG-Sepharose column.

2. Purification of HIV specific human antibody.

15 4 mls of pooled human HIV serum heat treated as outlined above was passed through a PD-10 column equilibrated with freshly prepared 0.05M Phosphate buffer pH7.2 + 0.5M NaCl. The first 3 mls fraction (void volume) was discarded and the next 7.5 mls was collected. 10 mls
20 of the gp120-Sepharose matrix and 7.5 mls of the equilibrated serum were mixed end-over-end for 2 hrs at RT. Following extensive washing HIV specific Ig's were desorbed using buffer containing 4M $MgCl_2$ pH8.3. Approximately, 2 mg of HIV specific Ig was obtained using
25 this method.

C. THE PRODUCTION OF MOUSE MONOCLONAL ANTIBODY TO THE HUMAN AB1.

The production of Mouse monoclonal antibodies firstly involves the induction of the antibodies either by
5 in vivo methods or by in vitro methods.

By way of illustration the in vitro method is described.

Two groups of Balb/c mice were used in this experiment. The first group consisted of mice which had
10 been tolerized to human IgG1. This had been achieved by injecting mice intraperitoneally, 7 days previously, with 10 milligrams of human IgG1. The second group consisted of untolerized mice.

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Mouse Ab2 antibodies were induced in the following
15 way. 1.3×10^8 mouse spleen cells were recovered and washed in the incubation medium (Iscoves DMEM medium containing 20% foetal calf serum (FCS), 40% thymus conditioned medium (TCM), 5×10^{-4} 2-mercaptoethanol, 4mML-glutamine 50 IU Penicillin and 50 IU streptomycin).
20 HIV specific human immunoglobulins at a concentration of 10 micrograms/ml incubation medium was added to the mouse spleen cells. The total volume used in the incubation of the spleen cells with human antibody varied between 10 and 15 mls. In this example, the incubation was allowed to
25 proceed for 7 days in a heated (37°C) CO₂ incubator.

Following incubation the cells were recovered for fusion to ether SP2, NS1 or X63-Ag*.653 mouse myeloma

cells. The viability of the spleen cells was found to vary between 70 and 99% and the viability of the myeloma was generally 99%. For the sake of illustration SP 2 mouse spleen cells were used though other cells such as rat or human myeloma cells could be used in this procedure. Spleen cells were fused to the myeloma cells using polyethylene glycol 1500/4000 (Boehringer/Mannheim) using standard procedures and following 24 hrs incubation in a CO₂ incubator at 37°C the hybrids were plated out in the incubation medium now containing HAT.

SCREENING OF HYBRID SUPERNATES FOR Ab2 SPECIFICITY

Screening for Ab2 was carried out by ELISA. Normal human IgG was coated onto the ELISA trays (1µg/ml in carbonate-bicarbonate buffer pH 9.6, 4°C, 16hrs). HIV specific immunogen was coated (0.6µg/ml) on a separate tray, and mouse supernatants were added in the usual way. Trays were blocked with 2% BSA in PBS-Tween 20 and the color was allowed to develop using ABTS substrate. HIV IgG was also fragmented using Pepsin beads and the trays coated with the F(ab)'2 to further determine the specificity of the mouse Ab2.

In the screening protocol, antibodies obtained from uninfected individuals and HIV-infected individuals were coated onto separate ELISA (screening) plates. If mouse Ab2 bound to both plates it was discarded. If it bound only to the plate containing normal human antibody, it was also discarded. If the Ab2 bound the HIV plate exclusively

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it was used as a vaccine candidate. Several such vaccine candidates (Ab2) were found.

D. RECOVERY OF ANTI-HIV ANTIBODY CLONES PRODUCED IN SITU AS A RESULT OF NATURAL INFECTION.

5 In addition to the serum HIV antibodies purified by the abovementioned methods it is possible to obtain the human Abl by Epstein-Barr virus (EBV) transformation of human B cells obtained from individuals exposed to the AIDS virus.

10 By way of illustration the following method was used. Human peripheral blood lymphocytes (PBL's) were diluted 1:1 in phosphate buffered saline and the red cells removed by centrifugation through a Ficoll-hypaque cushion (pharmacia).

15 The PBL's either depleted or not depleted of monocytes and lymphocytes using methods familiar to those skilled in the art, were then transformed using for example the EBV isolate B95-8 in sterile tissue culture media (RPMI-1640) + 5% FCS). In a simple example the
20 B95-8 isolate is made available as a supernate which is mixed with the monocyte/T cell depleted fraction enriched for the B lymphocytes. The cells are grown in this mixture, fed as required, and expanded in 96-well flat bottomed plates prior to fusion with the mouse myeloma
25 cell line such as X63-Ag*.653. Screening is by a commercially available HIV antibody ELISA. Cloning and feeding (Medium containing HAT/NT) is by the usual method

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except that non transformed will be selected out by feeding with 1 micromolar Oubain.

All these methods must be carried out in hybridoma facilities suitable for work involving HIV as virus may be shed under these conditions.

E. PRODUCTION OF HUMAN Abl USING IN VITRO IMMUNIZATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND/OR SPLENIC LYMPHOCYTES.

SCANNED # 10

HIV specific human Abl may also be obtained by in vitro immunization using whole virus or native, recombinant HIV antigens and antigens bound to nitrocellulose. According to one method $3-4 \times 10^4$ human PBL's or human splenic lymphocytes depleted of monocytes/T lymphocytes using L-Leucine methyl ester can be immunized with small amounts (1 nanogram - 10 micrograms) of HIV antigen. The human Abl are monoclonal when the techniques of hybridoma technology as outlined in D. are used. Human Abl obtained in this way may be used as the immunogen to produce the Ab2 by either in vivo or in vitro culture techniques using human cells or cells of other species as the human Abl would house the prototypic paratopes as defined by the foregoing.

Such variations as will be apparent to those skilled in the art from the teaching hereof are deemed to be within the scope of the invention herein disclosed.